Developing a Panel of Microsatellite Markers for Common Moorhen (*Gallinula chloropus*) as a Research Tool for Population Studies

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I hereby declare that I am the sole author of this thesis. It is the result of my own work and is not the outcome of work done in collaboration, nor has any of it been submitted elsewhere for another degree.

Signed:_____ Date:_____

Abstract

The common moorhen (*Gallinula chloropus*) sometimes exhibits brood parasitism – a parent manipulating a conspecific host to raise its offspring, relieving the parent of the encumbrance of parental investment. It has been observed that expression of this behavior may be affected by genetic relatedness in two distinct populations of common moorhen. The proposed hypothesis is that genetic relatedness between hosts and parasites has an effect on the evolution of brood parasitism and on the cost to the host of raising offspring not their own. It is expected that the population with higher brood parasitism will exhibit lower levels of genetic relatedness than the other.

With DNA samples for both populations, the project goal was to develop a panel of microsatellite markers as a tool to assess genetic relatedness; such a panel has never previously been produced for the common moorhen. A selection of markers developed for related species was screened in order to develop an optimized panel of microsatellite markers, selected from among those found to have >3 variable alleles when amplified in the common moorhen. These markers will be used to genotype members of each population to assess genetic relatedness among individuals. The data will then be used to determine whether there is an effect of relatedness on brood parasitism.

Of 47 primer pairs tested, 29 (62%) amplify a product in the common moorhen. Of the 10 best optimized primer sets, all were shown to be variable with each having no fewer than 3 alleles. Eight of the 10 loci were more variable in the larger population, based on samples tested so far.

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Finally, I would like to thank my mentor, Dr. Susan B. McRae, for her teaching, guidance, and inspiration before, during, and after this project. You have taught me nearly everything I now know about the techniques needed perform research like mine. I hope you are pleased with my work and that our lab can continue to build upon it in the future.

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Introduction

Conspecific Brood Parasitism

It has recently become apparent that many different animals use secretive behavioral strategies during mating and reproduction. Such stealthy behaviors result from complex trade-offs between foraging time, social competition, predator defense, environmental responses, and many other variables. They can often improve an individual's reproductive success at a reduced ecological cost, and therefore have deep implications for its survival and reproduction (Giné *et al.* 2011).

Conspecific brood parasitism (CBP) is the reproductive strategy of laying one or more eggs in the nest of another female in the same population (Andersson 2001). CBP removes the necessity of the brood parasite caring for the parasitic egg(s) and resulting young. Therefore, the parasitic female is able to enhance its own reproductive success at a reduced ecological cost. The 'host' female can accept the egg and care for the parasitic offspring, or recognize it as foreign and employ a counter-strategy, such as egg rejection. If it accepts the egg, the host bears this cost of increased parental input without the assurance of any benefit (Andersson 2001).

In some avian populations, CBP may not actually represent parasitism, but rather a highly specialized form of cooperative breeding within a population (Andersson & Eriksson 1982). Related individuals can employ strategies that enhance both individuals' success when done cooperatively, so the resulting question which much be raised is whether relatedness has an effect on the evolution of conspecific brood parasitism. According to the principles of kin selection (Hamilton 1964), females might have less to lose by accepting and caring for the parasitic young of a relative, because doing so could enhance the overall fitness of close relatives. Therefore, if hosts gain an indirect fitness benefit from caring for parasitic offspring,

this benefit could offset the cost of the parasitism itself (McRae 1995). It has indeed been shown that some birds, such as the Barrow's goldeneye (*Bucephala islandica*) show high rates of relatedness between hosts and parasites (Jaatinen *et. al.* 2011), though other species of waterfowl do not (Semel & Sherman 2001, Anderholm *et. al.* 2009).

Cryptic or sneaky behavioral strategies such as CBP are often difficult to study using only field methods; the link between these behaviors and genetic questions is even more difficult to find. Therefore, the value of using molecular methods applied to ecological questions about these strategies is a powerful way to study them. The link between CBP and genetic relatedness is a prime example of this. Such a link requires detailed genetic information on the relatedness of individuals within populations compared to the breeding behaviors of those individuals (McRae & Burke 1996).

Microsatellites

One of the most dependable and repeatable ways to study genetic variability is the use of polymorphic microsatellites. Microsatellites are hyper-variable, short-repeat motifs of non-coding DNA. Loci differ by the number of nucleotides repeated – usually 2, 3, or 4 nucleotides – and alleles vary according to the number of repeats. Since microsatellites are in non-coding regions, they do not produce any phenotypic response in individuals and have no effect on fitness. They are therefore considered neutral markers not subject to natural selection. Because of this, microsatellites are free to mutate in populations and often do so at a relatively high rate. Microsatellites are inherited from parents to offspring in a Mendelian fashion (Hancock 1996). Variation in these sequences is produced as a result of slippage errors during DNA replication, usually resulting in the addition of a unit at the end of the sequence. Such mutations are passed

on to offspring and contribute to the diversity of the population. Over time, multiple mutations lead to a variety of unique alleles at a given microsatellite locus (Selkoe & Toonen 2006).

These sequences in the genetic code were once regarded as inconsequential. However, since the development of the Polymerase Chain Reaction (PCR) as a technique to rapidly and efficiently amplify copies of DNA segments, they have been recognized as a powerful tool for examining population structure, gene flow, and genetic variability (Jarne & Lacoda 1996, Loyau & Schmeller 2009). Based on the frequencies of alleles at multiple loci, statistical probability based on maximum-likelihood methods can be applied to problems in behavioral ecology involving assignment of parentage, relatedness, and spatial questions regarding dispersal. Though powerful, microsatellite analysis requires the characterization for the species of interest of multiple loci that are highly variable (having multiple alleles) and that can be amplified reliably.

Microsatellites are located between segments of unique sequence (flanking regions) in the genome. Unique primers are designed in the flanking regions to either side of the microsatellites, and these are used to amplify a genomic DNA template using PCR (Selkoe & Toonen 2006). Characterizing new species-specific microsatellites is time-consuming and can be cost-prohibitive. However, many are published as freely-available resources that can be screened for use in other species. The probability of successful amplification and polymorphism from a related species is expected to be inversely related to the phylogenetic distance between the two species (Primmer *et. al*. 1996, 2005). Such cross-amplification techniques are common in avian studies because of time savings and reduced resource requirements for cross-amplification as opposed to cloning new species-specific markers from scratch. A very large number of loci have been identified for this purpose (Dawson *et. al* 2010).

Common Moorhen

The common moorhen (*Gallinula chloropus*) (hereafter "moorhen") has been shown to exhibit conspecific brood parasitism (McRae 1996). The moorhen is a water bird belonging to the rail family. It has a wide distribution across several continents. Moorhens are sexually monomorphic and mostly socially monogamous during breeding seasons, with pairs that often change in successive years. Females show higher incidence of competition for mates, while males are largely responsible for nest selection and building as well as defense against predators and other intruders (Loyau & Schmeller 2012). While males perform most of the incubation, moorhens show a combination of biparental care and semi-precocial young. Chicks are capable of leaving the nest one day after hatching but require feeding and other care from their parents for 3 to 6 weeks (McRae & Burke 1996, Loyau & Schmeller 2012).

The moorhen is an exceptional model species for CBP both because of its widespread distribution and because individual females lay eggs with conspicuously different shell patterns. It is sometimes possible to identify brood parasites in the field by examining egg patterns in each nest to find those that are distinguishable from the rest, and to use this information in combination with data on the sequence of egg-laying to identify parasitic eggs. However, there has been little or no conclusive evidence for the moorhen of a link between CBP and genetic relatedness. Also, there has been only a cursory attempt to develop a panel of microsatellites for the moorhen, and this turned out to be insufficient for population-level studies (Loyau & Schmeller 2012).

Project Goal

The goal of this project was to use cross-amplification of microsatellite markers developed from related species to identify and characterize a workable panel of polymorphic microsatellites for the common moorhen. In particular, for the first time, I screened multiple loci developed from two species of North American rail for use in the moorhen: markers for the king rail (*Rallus elegans*) developed in my lab (Brackett *et al.*, in prep) and markers for the black rail (*Laterallus jamaicensis*) (Girard *et al.* 2010). The resulting panel of markers will be used to determine relatedness of individuals within moorhen populations on two continents, one from Panama and one from Britain, for which DNA samples were already collected (McRae & Burke 1996; McRae 2011). While both populations exhibit brood parasitism, a higher proportion of nests were parasitized in Panama, and host rejection of parasitic eggs occurred in Panama while British hens did not show sophisticated egg rejection behavior. The Panamanian population is also smaller and more isolated than the British population. Based on kin selection theory, British hens may not be rejecting parasitic eggs because they are more related to the parasite females, and therefore the reduced cost of accepting parasitic eggs may result in insufficient selection pressure on hosts to evolve sophisticated egg rejection behavior. For these reasons, I hypothesize that the Panamanian population with higher rates of specific host rejection behavior will exhibit lower levels of genetic relatedness among neighbors than the British population.

Methods

DNA Sampling

This project utilized moorhen DNA samples from presumed unrelated individuals in a British and a Panamanian population collected as part of previous projects. These were obtained via 100-160 μL samples of blood by puncture of the brachial vein. Samples collected were from among adults and chicks from the British population and only chicks from the Panamanian population because of difficulties capturing the adults. For sample treatment, please see McRae & Burke (1996) and McRae (2011). DNA was phenol-chloroform extracted according to Maniatis *et al.* (1989). For samples containing 100 μL or more blood, the blood solution was proteinased in 3 ml TNE, and the DNA extracted by high salt precipitation (Miller *et al.* 1988; Bruford *et al.* 1992). Samples of embryonic tissue were ground in liquid nitrogen, and the DNA extracted by high salt precipitation. The DNA samples were stored at below -20 ^{o}C after extraction.

Cross-species Amplification and PCR Parameters

A total of 47 primer pairs were tested for cross-species amplification that had been characterized from the following species: 19 from black rail (*Laterallus jamaicensis*), 15 from king rail (*Rallus elegans*), and 11 from Tasmanian native-hen (*Gallinula mortierii*, formerly *Tribonyx mortierii*), 1 from Seychelles warbler (*Acrocephalus sechellensis*) , and 1 from transvolcanic jay (*Aphelocoma ultramarina*). The latter two loci were tested because a previous study that had screened other markers for moorhens found they amplified (Loyau, unpublished).

Microsatellite loci were tested for amplification using PCRs on PTC-100 (MJ Research) Biorad thermal cyclers. In order to arrive at an optimized set of PCR conditions for each primer

set, I first used the conditions found to be successful for the original species. If loci did not amplify sufficiently or showed excess subsidiary product, I used a gradient of annealing temperatures to find the optimum annealing temperature. Finally, I adjusted the magnesium chloride ($MgCl_2$) levels to adjust strength of amplification.

Each reaction consisted of a total of 10 µL of reagents. Reagents for each reaction included 1 µL (15-50 ng) of moorhen template DNA, 1 µL (50 mM) KCl buffer, 5 to 50 mM $MgCl_2$, 1 µL (0.2 mM) dNTPs, 0.5 µL (0.5 pmol) of each forward and reverse primer, and 0.1 units of *Taq* polymerase (Invitrogen or Bioline).

PCR programs used for all primers except the Tasmanian native-hen primers were as follows: 95°C for 2 minutes; 30 (35 for king rail primers) cycles of 95°C for 30 seconds, 55-59°C for 30 seconds, 72°C for 1 minute; and 72°C for 5 minutes (final extension).

PCR programs used for Tasmanian native-hen primers were as follows: 94°C for 2 minutes; 4 cycles of 92°C for 30 seconds, 60°C for 45 seconds with a drop of 1°C or 2°C per cycle; 72°C for 45 seconds; 25 cycles of 92°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute; and 72°C for 5 minutes (final extension).

The products of all PCRs were visualized by electrophoresis in a 2% agarose (w/v) gel containing 1.5×10^{-4} mg/mL ethidium bromide in a 1 x TBE buffer. This allowed determination of whether amplification occurred and rough estimation of the size of products amplified. A current of 70-80 V was applied for 45 minutes – 2 hours until products were sufficiently resolved. The resulting gel was visualized and image-captured under ultraviolet light using a Gel Logic 100 imaging system.

Variability Testing

Once amplifying primer pairs were identified, the PCR products of the best optimized loci were tested for variability using the Fragment Analysis module on an ABI 3130*xl* Genetic Analyzer (Applied Biosystems). Additional individuals were tested from each population in order to find as many separate alleles as possible at each locus. Primer pairs were labeled with fluorescent tags for this step. Fluorescent tags used were PET© (red) (Applied Biosystems), 6-FAM© (blue), and HEX© (green) (Bioneer). PCR products were multiplexed in 3 groups in order to allow simultaneous analysis of up to 5 loci with differently-colored fluorophores and/or products with very different size ranges. The products were diluted for multiplexing in relation to their estimated amplification strength as revealed by agarose gel electrophoresis. Products were visualized and sized via interpolation among fragments of a GS600 Liz© size standard using GeneMapper© (Applied Biosystems) software.

Results

A total of 47 microsatellite primer pairs were tested for cross-amplification in the common moorhen (Figure 1, Table 1). Twenty-nine (62%) of these successfully amplified a product detectable on agarose gel, including 11 (100%) from Tasmanian native-hen, 10 (67%) of 15 from king rail, 6 (32%) of 19 from black rail, 1 (100%) from Seychelles warbler, and 1 (100%) from transvolcanic jay.

Among the reliably amplifying loci, the 11 primer sets for which I was able to achieve best optimization (Table 2) – giving the clearest, sharpest products with the least amount of subsidiary product when visualized on agarose – were tested for variability using the genomic moorhen DNA of at least 31 individuals from each of the British and Panamanian populations (Figures 2 & 3). All of these 11 loci had more than one microsatellite allele in both populations. One of the 11 loci was later removed from further analysis because of substantial subsidiary product and lack of sufficient amplification strength when visualized via fragment analysis, though further PCR optimization is planned. All 10 remaining loci were shown to be variable (Table 3), having no fewer than 3 alleles in the British population; 8 (80%) had 3 or more alleles in the Panamanian population. Eight of the 10 loci were more variable in the British population than in the Panamanian population, based on the samples tested so far.

Figure 1 – Cross-amplification of the Tasmanian native-hen Tm 27 locus for 16 individuals from the British population as resolved on an agarose gel. The final well shows a 100 base-pair ladder added for the purpose of relative size approximations.

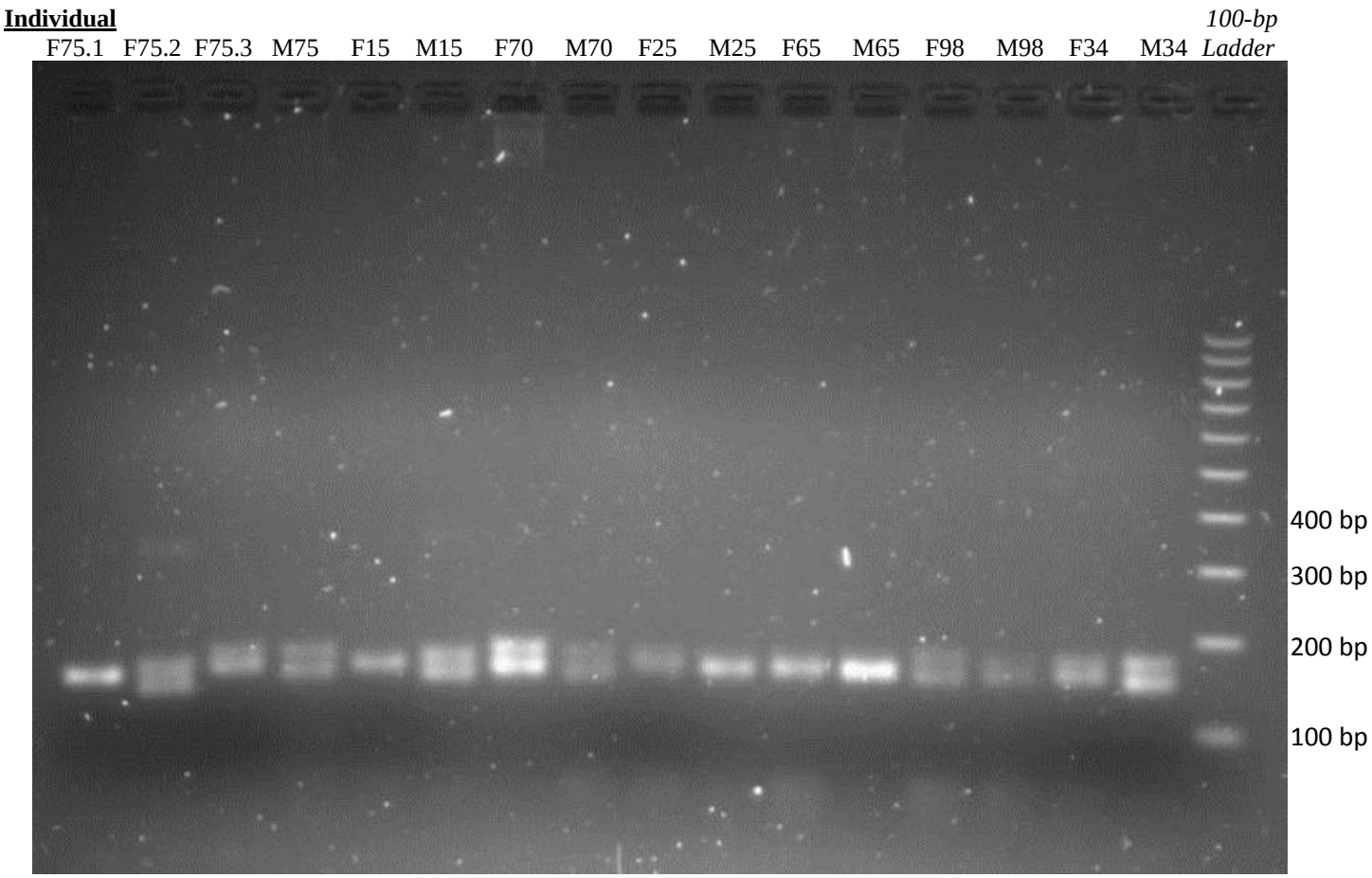


Table 1 – Results of cross-species amplification of avian primers using moorhen DNA

Interspecific Primer Pairs Tested						
Species of Origin	**Common Name**	**Primers Tested**	**Amplification Successes**	**% Amplification Success**	**Tested for Variability**	**Variability Successes**
Gallinula mortierii	Tasmanian Native-Hen	11	11	100.0	7	7
Rallus elegans	King Rail	15	10	66.7	4	4
Laterallus jamaicensis	Black Rail	19	6	31.6	0	0
Acrocephalus sechellensis	Seychelles Warbler	1	1	100	0	0
Aphelocoma ultramarina	Transvolcanic Jay	1	1	100	0	0
TOTAL		**47**	**29**	**61.7**	**11**	**11**

Table 2 – Optimized PCR parameters for the final 10 loci

PCR Parameters							
Primer Pair	**Species of Origin**	**Fluorescent Label Color**	**Panel**	**MgCl2/rxn (uL)**	**Ta (°C) (KiRa)**	**# Cycles (KiRa)**	**TD (°C) (Tm)**
***Tm* 18**	Tasmanian Native-hen	Blue	2	0.1			60 - 55
***Tm* 31b**	Tasmanian Native-hen	Blue	1	0.0			60 - 55
***Tm* 38**	Tasmanian Native-hen	Blue	1	0.0			60 - 55
KiRa 16	King Rail	Green	2	0.5	59	30	
***Tm* 105**	Tasmanian Native-hen	Green	1	0.1			60 - 50
***Tm* 27**	Tasmanian Native-hen	Green	3	0.1			60 - 50
***Tm* 36**	Tasmanian Native-hen	Green	2	0.2			60 - 50
KiRa 17	King Rail	Red	1	0.1	56	35	
KiRa 5	King Rail	Red	1	0.7	56	35	
***Tm* 20**	Tasmanian Native-hen	Red	2	0.1			60 - 55

Figure 2 – GeneMapper profiles of a multiplex of the Tm 18 (**blue**), Tm 105 (**green**), and KiRa 5 (**red**) loci for 2 individuals from the British population. For these 2 individuals, **Tm 18** produced 2 heterozygotes (2 major peaks) and 3 alleles total**. Tm 105** produced 2 heterozygotes and 3 alleles total. **KiRa 5** produced 1 homozygote (1 major peak) and 1 heterozygote, and 2 alleles total.

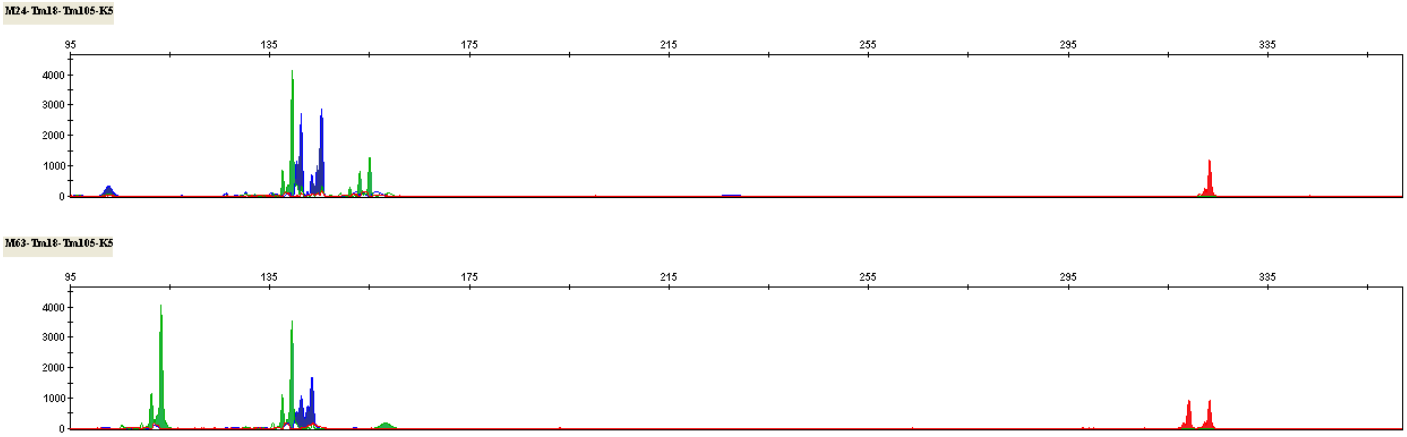


Figure 3 – GeneMapper profiles of the Tm 36 locus for 4 individuals from the British population. These 4 individuals produced 2 homozygotes and 2 heterozygotes, and 4 alleles total.

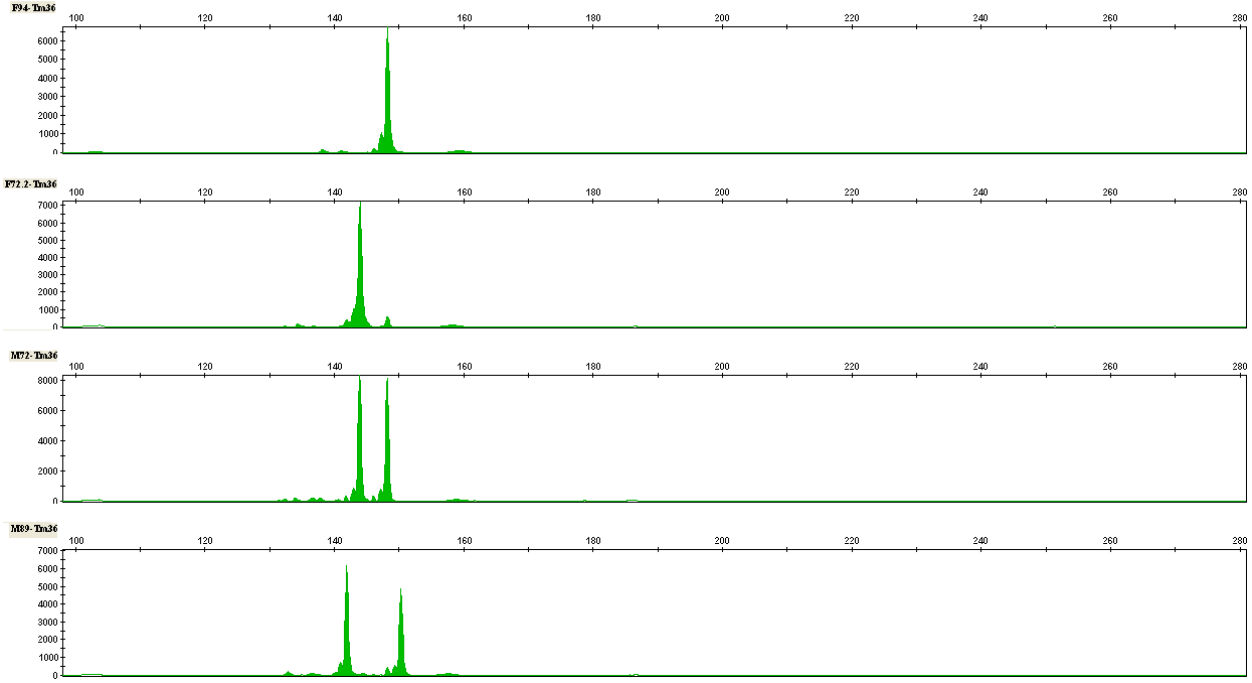


Table 3 – Variable microsatellite loci and their allele numbers and size ranges (in base pairs) in moorhens as determined from fragment analysis on an ABI 3130*xl* DNA Analyzer

Moorhen Variability							
		Britain Population			Panama Population		
Species of Origin	Locus	# Individuals Tested	# Alleles Total	Allele Range	# Individuals Tested	# Alleles Total	Allele Range
King Rail	KiRa 5	32	3	319 - 325	31	5	302 - 323
King Rail	KiRa 16	32	7	280 - 295	31	5	275 - 295
King Rail	KiRa 17	32	6	142 - 234	31	2	148 - 159
Tasmanian Native-Hen	Tm 18	32	5	139 - 149	31	3	132 - 143
Tasmanian Native-Hen	Tm 20	32	4	120 - 132	31	2	120 - 130
Tasmanian Native-Hen	Tm 27	32	10	132 - 157	31	5	144 - 153
Tasmanian Native-Hen	Tm 31b	32	15	190 - 261	31	9	177 - 201
Tasmanian Native-Hen	Tm 36	32	8	131 - 150	31	6	148 - 190
Tasmanian Native-Hen	Tm 38	32	7	108 - 148	31	4	102 - 150
Tasmanian Native-Hen	Tm 105	32	12	113 - 157	31	15	107 - 155

Moorhen Variability				
		Both Populations		
Species of Origin	Locus	# Individuals Tested	# Alleles Total	Allele Range
King Rail	KiRa 5	63	6	301 - 325
King Rail	KiRa 16	63	8	275 - 295
King Rail	KiRa 17	63	6	142 - 234
Tasmanian Native-Hen	Tm 18	63	6	132 - 149
Tasmanian Native-Hen	Tm 20	63	4	120 - 132
Tasmanian Native-Hen	Tm 27	63	10	132 - 157
Tasmanian Native-Hen	Tm 31b	63	22	177 - 261
Tasmanian Native-Hen	Tm 36	63	12	131 - 190
Tasmanian Native-Hen	Tm 38	63	9	102 - 150
Tasmanian Native-Hen	Tm 105	63	18	107 - 157

Discussion

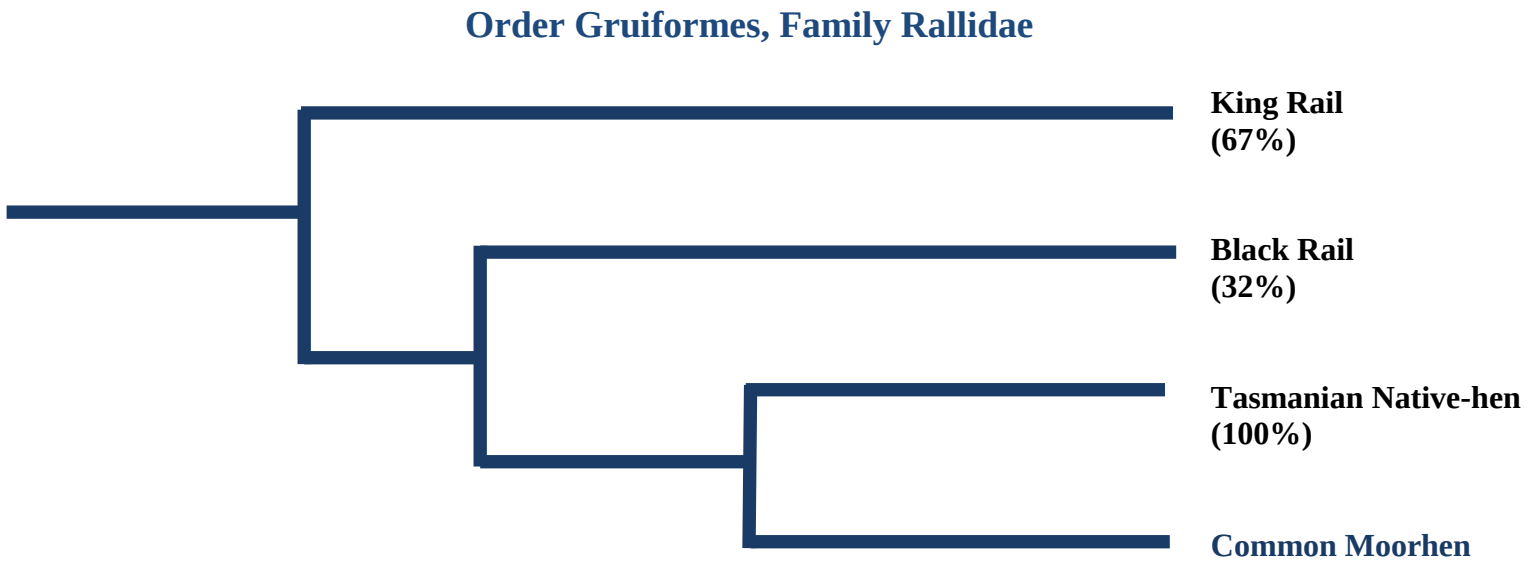
Cross-Species Amplification and Variability

My project tested 47 interspecific primer pairs for amplification of microsatellite loci in the common moorhen, and I found 27 that amplified successfully. I also optimized PCR conditions and analyzed the variability of 10 of these loci. Each exhibited no fewer than 3 alleles in the British moorhen population. Eight of these loci showed 3 or more alleles in the Panamanian population, and the remaining 2 each showed 2 alleles. In combination, these loci will be useful for parentage and population genetic studies in the moorhen.

One hundred percent of the Tasmanian Native-hen (*Gallinula mortierii*) loci showed successful cross-species amplification in the moorhen, followed by 67% of the king rail loci and 32% of the black rail loci. The native-hen is from the same genus as the moorhen and the king rail and black rail are both from the same family (Rallidae). These results support the idea that amplification success and polymorphism from a related species is expected to be inversely related to the phylogenetic distance between the two species. This is because microsatellites are more likely to be conserved in the genomes of closely-related species (Primmer *et al.* 1996). Figure 4 shows a simplified phylogenetic tree for these 4 species (after Livezey 1998) and the percent cross-species amplification success for each species' loci in the common moorhen.

This idea is further supported by the results from variability testing of these loci. Seven of the 10 variable loci for the British population (6 of the 8 for the Panamanian population) were originally characterized from the Tasmanian native-hen. The remaining 3 variable loci for the British population (2 from the Panamanian population) were originally from the king rail, which is from the same family as the moorhen. The PCR conditions for the black rail primers could not easily be optimized due to significant subsidiary product and unbound primer dimer after the

Figure 4 – Phylogenetic tree of the rail family simplified to show species of origin for the microsatellite loci in relation to the common moorhen. Percent amplification success for loci in the moorhen is indicated in parentheses.



PCR was complete.

One locus from the Seychelles warbler (*Acrocephalus sechellensis*) and 1 from the transvolcanic jay (*Aphelocoma ultramarina*) were also tested for amplification because a previous study found that they amplified successfully (Loyau, unpublished). This project found that they amplified successfully in the moorhen as well. However, the PCR conditions for these primers also could not be optimized and therefore variability could not be determined.

The panel of 10 variable microsatellites produced from this project is expected to be useful in parentage and population studies of the common moorhen and should aid in the determination of relatedness, levels of gene flow and genetic variability among populations, and general population structure (Jarne & Lacoda 1996, Loyau & Schmeller 2009). Future research should expand upon these results by attempting to further optimize moorhen PCR conditions for the primers from the black rail, Seychelles warbler, and transvolcanic jay, and then testing them for variability in the moorhen.

Population Studies and CBP

It was observed that, for 8 of the 10 loci studied, the British population exhibited more alleles than the Panamanian population. The imbalance will make it more difficult to compare average relatedness between populations, for example in testing the hypothesis that neighbors are more closely related in Britain than in Panama. Such variability results do assent with the field observation that the British population is larger and likely older than the Panamanian population, and therefore the alleles in the Panamanian population have not had as much opportunity to diverge.

The panel of microsatellite markers produced during this project will be used to genotype individual moorhens from the British and Panamanian populations. The resulting data will then be compared to field data gathered for each population, particularly spatial dispersion data. Since a higher proportion of nests were parasitized in Panama, and host rejection of parasitic eggs occurred in Panama while British hens did not show sophisticated egg rejection behavior, these data will be used to determine the relationship, if any, between relatedness and brood parasitism and between relatedness and egg rejection patterns in the moorhen. If there is a significant relationship, this could generate a number of predictions regarding moorhen population and ecological strategies. According to Jaatinen *et. al.* (2011), some of these might include that (i) parasites who are related to neighboring hosts might have better information about them through shared previous life experience, so are more effective in parasitizing them; (ii) parasites who are related to neighboring hosts might show more closely clumped distributions with their hosts, so parasites have less distance to travel to parasitize; or (iii) parasites who are related to their hosts are actually employing a consensual and cooperative breeding strategy – therefore the behavior is not actually parasitic. In this latter hypothesis, females should be less likely to reject parasitic eggs of relatives than those of non-relatives because of the indirect fitness benefit to the host from raising related offspring, and because of the benefit to the parasite from recovering additional energy that could be used to lay more eggs.

If the latter hypothesis is supported, this would also raise questions regarding kin selection – how moorhens manage to recognize their relatives for parasitic activity. Possibilities for this include (i) familiarity through previous life experience; (ii) shared traits among kin such as timing of breeding or nest site preferences, or (iii) in the absence of previous experience, some shared, environmentally-stimulated phenotype (Eadie & Lyon 2011).

The microsatellite panel created for this project could also be used to examine the genetic basis of other moorhen behaviors as well as population structure in the moorhen at regional scales. For example, the moorhen is a hunted species in the U.S. and recent declines in wetland habitat have caused population declines in this and related species that may necessitate future monitoring programs (Baldwin and Batzer 2012).

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